

## PATENT APPLICATION TRANSMITTAL LETTER

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Address to: Commissioner of Patents and Trademarks  
Washington DC 20231

Transmitted herewith for filing is the patent application of

Inventors: Hiroyuki NAKANE, Chikara OHTO, Shinichi OHNUMA, Kazutake HIROOKA  
and Tokuzo NISHINO

For: Farnesyl Diphosphate Synthase

Enclosed are:

1. 29 pages of written specification, 3 pages of claims, 3 pages of drawings, Abstract
2. Executed Declaration and Power of Attorney
3. Executed Assignment to: Toyota Jidosha Kabushiki Kaisha, Aichi, JAPAN
4. Certified copy of Japanese Application No. 8-213211, filed July 24, 1996
5. Preliminary Amendment
6. Sequence Listing - Computer Readable Copy and Paper Copy,  
with Verification Statement under 37 CFR §1.821(f)

	TOTAL FILED	# EXTRA	RATE (\$)	FEE (\$)
BASIC FEE			770.00	770.00
TOTAL CLAIMS	26 - 20	6	22.00	132.00
INDEPENDENT CLAIMS	1 - 3	0	80.00	- 0 -
MULTIPLE DEPENDENT CLAIM			260.00	260.00
			TOTAL	\$1,162.00
			SMALL ENTITY TOTAL	\$

Please charge the filing fee of \$1,162.00 to Deposit Account 11-0600.  
A duplicate copy of this sheet is enclosed for that purpose.

The Commissioner is hereby authorized to charge payment of the following fees associated with this communication or credit any overpayment to the Deposit Account No. 11-0600.

- a. Any additional filing fees required under 37 C.F.R. § 1.16.
- b. Any patent application processing fees under 37 C.F.R. § 1.17.

Respectfully submitted,

Date July 22, 1997

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**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Inventors: Hiroyuki NAKANE, et al.

Art Unit: -- Not Yet Assigned --

Serial No.: -- Not Yet Assigned --

Filing Date: -- Concurrent Herewith --

For: FARNESYL DIPHOSPHATE SYNTHASE

Commissioner of Patents and Trademarks  
Washington, DC 20231

Sir:

**PRELIMINARY AMENDMENT**

Prior to examination of the subject application on the merits, please amend the same as follows.

**In the Specification**

Please amend the specification as follows.

Pages 25, 26, 27, 28 and 29, discard these pages and replace with the 8 pages of Sequence Listing attached hereto, numbering the 8 pages of the Sequence Listing 25, 26, 27, 28, 29, 30, 31 and 32.

Page 30, containing Claims 1 through 7, renumber as page 33.

Page 31, containing Claims 8 through 12, renumber as page 34.

Page 32, containing Claims 13 through 16, renumber as page 35.

Page 33, containing the Abstract, delete the page number.

**REMARKS**

Claims 1 to 16 are pending in the present application.

The specification has been amended to insert a substitute copy of the Sequence Listing that complies with formatting requirements. The computer readable copy of the Sequence Listing and the paper copy of the Sequence Listing are the same, and do not add new matter to the specification.

It is respectfully submitted that the application, as amended, is in condition for examination on the merits.

The Commissioner is authorized to charge any fees relevant to this filing to Kenyon & Kenyon Deposit Account 11-0600.

Respectfully submitted,

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FARNESYL DIPHOSPHATE SYNTHASE

## BACKGROUND OF INVENTION

## 5 1. Field of Invention

The present invention relates to a novel mutant enzyme which synthesizes linear prenyl diphosphates that are precursors of compounds, important for organisms, such as steroids, ubiquinones, dolichols, carotenoids, 10 prenylated proteins, animal hormones, plant hormones, and the like; a genetic system encoding said enzyme; and a method for producing and using said enzyme.

## 2. Related Art

Of the substances having important functions in 15 organisms, many are biosynthesized using isoprene (2-methyl-1,3-butadiene) as a constituent units. These compounds are also called isoprenoids, terpenoids, or terpenes, and are classified depending on the number of carbon atoms into hemiterpenes (C5), monoterpenes (C10), 20 sesquiterpenes (C15), diterpenes (C20), sesterterpenes (C25), triterpenes (C30), tetraterpenes (C40), and the like. The actual biosynthesis starts with the mevalonate pathway through which mevalonic acid-5-diphosphate is synthesized, followed by the synthesis of isopentenyl 25 diphosphate (IPP) which is an active isoprene unit.

The identity of the isoprene unit that was proposed as a precursor was found to be isopentenyl diphosphate, the so-called active isoprene unit.

Dimethylallyl diphosphate (DMAPP), an isomer of 30 isopentenyl diphosphate, being used as a substrate in the synthesis of isopentenyl adenine which is known as a cytokinin, one of the plant hormones, it is also known to undergo a condensation reaction with isopentenyl diphosphate to synthesize chain-form active isoprenoids 35 such as geranyl diphosphate (GPP), neryl diphosphate, farnesyl diphosphate (FPP), geranylgeranyl diphosphate (GGPP), geranylgeranyl diphosphate (GFPP), hexaprenyl

diphosphate (HexPP), heptaprenyl diphosphate (HepPP), and the like.

There are Z type and E type condensation reactions. Geranyl diphosphate is a product of E type condensation and neryl diphosphate is of Z type condensation. Although, the all-E type is considered to be the active form in farnesyl diphosphate and geranylgeranyl diphosphate, the Z type condensation reaction leads to the synthesis of natural rubber, dolichols, bactoprenols (undecaprenols), and plants various polyprenols found in. They are believed to undergo the condensation reaction using the phosphate ester bond energy of the pyrophosphate and the carbon backbone present in the molecule and to produce pyrophosphate as the byproduct of the reaction.

Farnesyl diphosphate or geranylgeranyl diphosphate serve as a reaction substrate leading to the synthesis of prenylated proteins (from farnesyl diphosphate or geranylgeranyl diphosphate) represented by G proteins that are important in the mechanism of signal transducer in the cell; cell membrane lipids (from geranylgeranyl diphosphate) of archaea; squalene (from farnesyl diphosphate) which is a precursor of steroids; and phytoene (from geranylgeranyl diphosphate) which is a precursor of carotenoids. Prenyl diphosphates from hexaprenyl diphosphate and heptaprenyl diphosphate having six and seven isoprene units, respectively, to prenyl diphosphates having ten isoprene units serve as the precursors of the synthesis of ubiquinone and menaquinone (vitamin K2) that work in the electron transport system.

Furthermore, via the biosynthesis of these active-form isoprenoids, a vast number of kinds of compounds that are vital to life have been synthesized. Just to mention a few, there are cytokinins that are plant hormones and isopentenyl adenosine-modified tRNA that use hemiterpenes as their precursor of synthesis, geraniols and that isomer nerol belonging to monoterpenes

are the main components of rose oil perfume and a camphor tree extract, camphor, which is an insecticide.

Sesquihormones include juvenile hormones of insects, diterpenes include a plant hormone gibberellin, trail 5 pheromones of insects, and retinols and retinals that function as the visual pigment precursors, binding components of the purple membrane proteins of highly halophilic archaea, and vitamin A.

Furthermore, using squalene, a triterpene, a wide variety of steroid compounds have been synthesized, including, for example, animal sex hormones, vitamin D, 10 ecdisone which is an ecdisis hormone of insects, a plant hormone brassinolide, constitution of the plasma membrane etc. Various carotenoids of tetraterpenes that are 15 precursors of various pigments of organisms and vitamin A are also important compounds derived from active isoprenoids. Compounds such as chlorophyll, pheophytin, tocopherol (vitamin E), and phylloquinone (vitamin K1) are also derived from tetraterpenes.

20 The active isoprenoid synthases that sequentially condense isopentenyl diphosphates with such allylic substrates as dimethylallyl diphosphate, geranyl diphosphate, farnesyl diphosphate, geranylgeranyl 25 diphosphate, geranylgeranyl diphosphate, etc. are called the prenyl diphosphate synthases, and are also called, based on the name of the compound having the maximum chain length of the major reaction products, for example 30 farnesyl diphosphate synthase (FPP synthase), geranylgeranyl diphosphate synthase (GGPP synthase), and the like.

35 There are reports on purification, activity measurement, genetic cloning, and sequencing of the DNA encoding enzymes such as farnesyl diphosphate synthase, geranylgeranyl diphosphate synthase, hexaprenyl diphosphate synthase, heptaprenyl diphosphate synthase, octaprenyl diphosphate synthase, nonaprenyl diphosphate synthase (solanesyl diphosphate synthase), undecaprenyl diphosphate synthase, and the like from bacteria,

archaea, fungi, plants, and animals.

These active isoprenoid synthases constituting the basis of chemical synthesis of a great variety of compounds that are important both in the industry and in the academic field of life sciences have had few practical uses in the industrial application due to their unstable nature and low specific activities. However, with the isolation of thermostable prenyl diphosphate synthases from thermophilic bacteria and archaea and the genes encoding these enzymes, their availability as the enzyme has increased.

With regard to farnesyl diphosphate synthase, a gene was isolated from Bacillus stearothermophilus, a medium thermophile, and an enzyme having a medium thermal stability was prepared using Escherichia coli as host cell [T. Koyama et al. (1993) J. Biochem., 113: 355-363; Japanese Unexamined Patent Publication No.

5(1993)-219961]. With regard to geranylgeranyl diphosphate synthase, a gene was isolated from high thermophiles such as Sulfolobus acidocaldarius and Thermus thermophilus [S. -i. Ohnuma et al., (1994) J. Biol. Chem., 269: 14792-14797; Japanese Unexamined Patent Publication No. 7(1995)-308193, and; Japanese Unexamined Patent Publication No. 7(1995)-294956], and enzymes having a high thermal stability were prepared.

Furthermore, with regard to the prenyl diphosphate synthase having the functions of both of the farnesyl diphosphate synthase and the geranylgeranyl diphosphate synthase, the enzyme and the gene encoding it have been isolated from highly thermophile Methanobacterium thermoautotrophicum [A. Chen and D. Poulter (1993) J. Biol. Chem., 268: 11002-11007; A. Chen and D. Poulter (1994) ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS 314], and the thermostable nature of the enzyme has been demonstrated.

However, in the synthesis of farnesyl diphosphate/geranylgeranyl diphosphate derived from

5        Methanobacterium thermoautotrophicum, there are no reports on the data of thin layer chromatography analysis etc. that can specify the chain length of the reaction products in connection with the assay of the enzymatic activity; the chain length has been estimated by measuring geranyl diphosphate as the allylic substrate. Since geranyl diphosphate can also serve as a substrate of geranylgeranyl diphosphate synthase, it is unlikely that the measured activity includes that of the farnesyl 10 diphosphate synthase alone.

15        Moreover, the presence of farnesyl diphosphate synthase has not been confirmed in archaea that are expected to have enzymes having higher thermo stability, higher salt-stability and lower-pH-stability.

20        As mentioned above, the use of the farnesyl diphosphate synthase derived from Bacillus stearothermophilus resolved part of the problem of the enzyme being unstable and difficult to handle. But, an enzyme having a higher thermal stability would be more stable and more amenable to industrial application.

25        Moreover, some prenyl diphosphate synthases having a longer chain length use farnesyl diphosphate as a substrate. When such a long-chain prenyl diphosphate synthase is used simultaneously with a farnesyl diphosphate synthase for the purpose of providing the substrate of the former enzyme, the latter enzyme must have stability which is equal to or higher than that of the long-chain prenyl diphosphate synthase. When industrial production of farnesyl diphosphate is 30 contemplated, the enzyme must be immobilized or recovered for recycling. When it is regenerated, the enzyme itself to be more stable, must have higher thermo stability, higher salt stability, and higher stability in a wider range of pH.

35        It has been found out that of the two aspartic acid-rich domains that have been proposed based on the amino acid sequence of the prenyl diphosphate synthase,

the amino acid residue located at the fifth position in the N-terminal direction from the conserved sequence I (DDXX(XX)D) (wherein X denotes any amino acid, and the two X's in the parentheses may not be present) of the 5 aspartic acid-rich domain in the amino-terminal side is responsible for controlling the chain length of the reaction product. Hence, a method has been invented that controls the reaction product for the purpose of lengthening the chain length of the reaction product 10 [Japanese patent application No. 8-191635 filed on July 3, 1996 under the title of "A Mutant Prenyl Diphosphate Synthase"]. The enzyme produced using the method enables production of reaction products that have several chain 15 lengths. However, methods have not been known that induce mutation of geranylgeranyl diphosphate synthase to control the reaction products to be in the short chain-length side in order to produce farnesyl diphosphate.

#### SUMMARY OF INVENTION

20 It is an object of the invention to establish a process for producing farnesyl diphosphate synthases by modifying amino acid sequences of prenyl diphosphate enzymes. A new enzyme that is more stable or that has a high specific activity more adaptable to industrial 25 application would make it possible to obtain immediately a mutant prenyl diphosphate synthase or the gene thereof that produces farnesyl diphosphate and that retains the property owned by the the prenyl diphosphate synthase prior to mutation.

30 From the information on the nucleotide sequence of the gene of the geranylgeranyl diphosphate synthase of the mutant Sulfolobus acidocaldarius (*S. acidocaldarius*), it was clarified that out of the two Aspartic acid-rich domains that have been proposed based on the analysis of 35 the amino acid sequence of prenyl diphosphate synthases, the amino acid residues within the aspartic acid-rich domain conserved sequence I (DDXX(XX)D) at the amino

terminal side or the five amino acid residues to the N-terminal side from the amino terminal of said conserved sequence I are involved in the control of chain length of the reaction products.

5        Thus, the present invention provides a mutant prenyl diphosphate synthase having a modified amino acid sequence, wherein

10        at least one amino acid residue selected from (a) the amino acid residues in between the amino acid residue located at the fifth position in the N-terminal direction from D of the N-terminal and the amino acid residue located at the first position in the N-terminal direction from D of said N-terminal of the aspartic acid-rich domain DDXX(XX)D (wherein X sequence denotes any amino acid, and the two X's in the parentheses may not be present) present in region II, and (b) the amino acid residue located at the position in the N-terminal direction from D of the C-terminal of said aspartic acid-rich domain has been substituted by another amino acid, and/or

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20        additional amino acid(s) have been inserted in between the amino acid residue located at the first position in the N-terminal direction from D of the C-terminal and D of said C-terminal of said aspartic acid-rich domain.

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30        The present invention provides a farnesyl diphosphate-producing mutant prenyl diphosphate synthase which retains the properties that were owned by the native prenyl diphosphate synthase.

35        The present invention also provides a DNA or an RNA encoding the above enzyme.

40        The present invention further provides a recombinant vector and more specifically an expression vector comprising the above DNA.

45        The present invention further provides a host transformed by the above vector.

50        The present invention further provides a process for

producing prenyl diphosphates having not more than 15 carbons comprising the step wherein the above enzyme is brought into contact with a substrate selected from the group consisting of isopentenyl diphosphate, dimethylallyl diphosphate, and geranyl diphosphate.

The present invention further provides a process of production of a mutant enzyme according to any of claims 1 to 8, said method comprising the steps of culturing the above host and of harvesting the expression product from the culture.

#### BRIEF EXPLANATION OF THE DRAWINGS

Fig. 1 is a graph showing the regions (I) to (V) and the aspartic acid-rich domain I of various prenyl diphosphate synthases. In the figure, the sequence represents the amino acid sequence of geranylgeranyl diphosphate synthase, and ATGERPYRS is the one derived from Arabidopsis thaliana, LA15778.p from Lupinus albus, CAGERDIS from Capsicum annuum, ATGGPSRP from Arabidopsis thaliana, GGPS-pep from Sulfolobus acidocaldarius, SPCRT.pep from Rhodobacter sphaeroides, RCPHSYNG from Rhodobacter capsulatus, EHCRTS.pe from Erwinia herbicola, MXCRTNODA from Myxococcus thaliana, and NCAL3.pep from Neurospora crassa. The number indicated on the left of each amino acid sequence represents the site from the N-terminal side of each geranylgeranyl diphosphate synthase at the N-terminal of the amino acid sequence.

Fig. 2 is a graph showing the thermal stability of the mutant prenyl diphosphate synthase. The ordinate shows the relative activity to 100% at incubation at 60°C. The abscissa shows the incubation temperature. SacGGPS is the geranylgeranyl diphosphate synthase prior to mutation. The others represent the mutant type enzyme of each. BstFPS is the farnesyl diphosphate synthase derived from Bacillus stearothermophilus.

Fig. 3 shows a photograph of a development pattern of thin layer chromatography of the dephosphorylated reaction products of the mutant prenyl diphosphate

synthase when geranyl diphosphate was used as the allylic substrate. In the figure, ori. represents the origin of development, and s.f. represents the solvent front.

5 GOH is geraniol, FOH is farnesol, GGOH is geranyl geraniol, and GFOH is geranyl farnesol, and these are produced from dephosphorylation of geranyl diphosphate, farnesyl phosphate, geranylgeranyl diphosphate, and geranyl farnesyl diphosphate, respectively. SacGGPS is the geranylgeranyl diphosphate synthase prior to 10 mutation. The others are each mutant enzymes.

#### DETAILED DESCRIPTION

It has been proposed that there are five conserved regions in the amino acid sequence of a prenyl diphosphate synthase (one subunit in the case of a 15 heterodimer) [A. Chem et al., Protein Science Vol. 3, pp. 600-607, 1994]. It is also known that of the five conserved regions, there is an aspartic acid-rich domain 20 conserved sequence I [DDXX(XX)D] (wherein X denotes any amino acid, and the two X's in the parentheses may not be present) in region II. Although there is also an aspartic acid-rich domain indicated as "DDXXD" in region V, the aspartic acid-rich domain used to specify the modified region of the amino acid sequence of the present invention is the one present in region II, and this 25 domain is termed as the aspartic acid-rich domain I as compared to the aspartic acid-rich domain II present in region V.

As the prenyl diphosphate synthases having the aspartic acid-rich domain as described above, there can 30 be mentioned farnesyl diphosphate synthase, geranylgeranyl diphosphate synthase, hexaprenyl diphosphate synthase, heptaprenyl diphosphate synthase, octaprenyl diphosphate synthase, nonaprenyl diphosphate synthase, undecaprenyl diphosphate synthase, and the 35 like. More specific examples include the farnesyl diphosphate synthase of Bacillus stearothermophilus, the farnesyl diphosphate synthase of Escherichia coli, the

farnesyl diphosphate synthase of Saccharomyces cerevisiae, the farnesyl diphosphate synthase of the rat, the farnesyl diphosphate synthase of the human, the geranylgeranyl diphosphate synthase of Neurospora crassa,  
5 the hexaprenyl diphosphate synthase of Saccharomyces cerevisiae, and the like.

By way of example of some of these, regions I to V and the aspartic acid-rich domain I (in the box) in region II of the amino acid sequence of geranylgeranyl diphosphate synthases are shown in Fig. 1.  
10

The present invention can be applied to any prenyl diphosphate synthase having the aspartic acid-rich domain I.

In accordance with the present invention, in the amino acid sequence of a prenyl diphosphate synthase, at least one amino acid residue selected from (a) the amino acid residues in between the amino acid residue located at the fifth position in the N-terminal direction from D of the N-terminal and the amino acid residue located at the first position in the N-terminal direction from D of said N-terminal of the aspartic acid-rich domain DD<sub>2</sub>XX(XX)D (wherein X denotes any amino acid, and the two X's in the parentheses may not be present) present in region II, and (b) the amino acid residue located at the first position in the N-terminal direction from D of the C-terminal of said aspartic acid-rich domain has been substituted by another amino acid, and/or  
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an additional one or more amino acids have been inserted in between the amino acid residue located at the first position in the N-terminal side from D of the C-terminal and D of said C-terminal of said aspartic acid-rich domain.  
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The mutant prenyl diphosphate synthase of the present invention can synthesize a farnesyl diphosphate having a shorter chain length than the prenyl diphosphate synthesized by the native prenyl diphosphate synthase.  
35

In accordance with the present invention, by way of

example, the gene of the geranylgeranyl diphosphate synthase of a highly thermophilic archaea, Sulfolobus acidocaldarius, is used as the starting material.

5 Sulfolobus acidocaldarius is available from ATCC as ATCC No. 33909. The method for cloning the gene has been described in detail in Japanese Unexamined Patent Publication No. 7-308193. It has also been disclosed with the accession No. D28748 in the gene information data base such as GenBank. By using the sequence it can  
10 be cloned in the conventional method known in the art. An example of the other cloning methods is illustrated in Example 1 herein and its nucleotide sequence is shown as SEQ ID No: 2.

15 More specifically, the mutant enzyme of the present invention is a mutant prenyl diphosphate synthase characterized in that at least one amino acid selected from phenylalanine in position 77, threonine at position 78, valine at position 80, histidine at position 81, and isoleucine at position 84 has been substituted by another  
20 amino acid, and/or amino acid(s) have been inserted in between isoleucine at position 84 and methionine at position 85 in the geranylgeranyl diphosphate synthase having the amino acid sequence as set forth in SEQ ID No: 1.

25 By way of example, there is provided the amino acid sequences wherein the amino acids have been substituted as shown below:

30 Mutant enzyme 1: Changes from threonine at position 78 to phenylalanine, and histidine at position 81 to alanine;

Mutant enzyme 2: Changes from threonine at position 78 to phenylalanine, and histidine at position 81 to leucine;

35 Mutant enzyme 3: Changes from phenylalanine at position 77 to tyrosine, threonine at position 78 to phenylalanine, and histidine at position 81 to leucine;

Mutant enzyme 4: Changes from phenylalanine at

position 77 to tyrosine, threonine at position 78 to phenylalanine, and histidine at position 81 to alanine;

Mutant enzyme 5: Changes from phenylalanine at position 77 to tyrosine, threonine at position 78 to serine, valine at position 80 to isoleucine, and isoleucine at position 84 to leucine, and an insertion of proline and serine in between isoleucine at position 84 and methionine at position 85.

In accordance with the present invention, it is indicated that the mutant prenyl diphosphate synthase retains the characteristic properties that were owned by the native prenyl diphosphate synthase. By way of example, the above-mentioned five mutant enzymes show thermo resistance almost equal to that owned by the native geranylgeranyl diphosphate synthase.

It is known that an enzyme can sometimes exhibit its original enzymatic activity even when it has been modified by addition, removal, and/or substitution of one or a few amino acids as compared to the original amino acid sequence. Therefore, the present invention is intended to encompass those enzymes that have been modified by addition, deletion, and/or substitution of one or a few, for example up to five, or up to 10, amino acids as compared to the amino acid sequence as set forth in SEQ ID No: 1 and that can perform its original function.

The present invention also provides the genes encoding various mutant enzymes mentioned above, the vectors containing those genes, specifically expression vectors, and the hosts transformed by said vectors. The gene (DNA) of the present invention can be readily obtained, for example, by introducing mutation into the DNA encoding the original amino acid sequence as set forth in SEQ ID No: 1 using a conventional method such as site-directed mutagenesis, PCR and the like.

Furthermore, once the amino acid sequence of the desired enzyme has been determined, an appropriate

nucleotide sequence encoding it can be determined, and the DNA can be chemically synthesized in accordance with a conventional method of DNA synthesis.

5 The present invention further provides an expression vector comprising DNA such as the one mentioned above, the host transformed by said expression vector, and a method for producing the enzyme or peptide of the present invention using these hosts.

10 Expression vectors contain an origin of replication, expression regulatory sequences etc., but they may differ depending on hosts used. As the hosts, there are mentioned procaryotes, for example, bacteria such as Escherichia coli, organisms of genus Bacillus such as Bacillus subtilis, and eukaryotic microorganisms, for example, fungi, for example yeast, for example Saccharomyces cerevisiae of genus Saccharomyces and Pichia pastoris of genus Pichia, filamentous fungi, for example the genus Aspergillus such as Aspergillus niger, animal cells, for example the cultured cells of the silkworm, cultured cells of higher animals, for example CHO cells, and the like. Furthermore, plants may also be used as the host.

25 As set forth in Examples, in accordance with the present invention, by cultivating the host transformed by the DNA of the present invention, farnesyl diphosphates may be accumulated in the culture broth, which may be harvested to produce their farnesyl diphosphates. Furthermore, in accordance with the invention, farnesyl diphosphates may also be produced by contacting the 30 mutant prenyl diphosphate synthase produced by the method of the invention to the substrate isopentenyl diphosphate and each allyl substrate such as dimethylallyl diphosphate and geranyl diphosphate.

35 When Escherichia coli is used as the host, it is known that the host has the regulatory functions at the stage of transcribing mRNA from DNA and of translating protein from mRNA. As the promoter sequence regulating

mRNA synthesis, in addition to the naturally occurring sequences (for example, lac, trp, bla, lpp,  $P_L$ ,  $P_R$ , ter, T3, T7, etc.), there are known their mutants (for example, lac UV5), and the sequences (such as tac, trc, etc.) in which a naturally occurring promoter is artificially fused, and they can be used for the present invention.

It is known that the distance between the sequence of the ribosome bidding site (GGAGG and similar sequences thereof) and the initiation codon ATG is important as the sequence regulating the ability of synthesizing protein from mRNA. It is also well known that a terminator (for example, a vector containing rrn  $P_{T_1} T_2$  is commercially available from Pharmacia) that directs transcription termination at the 3'-end affects the efficiency of protein synthesis by a recombinant.

As the vectors that can be used for preparation of the recombinant vectors of the present invention, commercially available vectors are used as they are, or various vectors may be mentioned that are derived depending on the intended use. For example, there can be mentioned pBR322, pBR327, pKK223-3, pKK233-3, pTrc99, and the like having a replicon derived from pMB1; pUC18, pUC19, pUC118, pUC119, pTV118N, pTV119N, pBluescript, pHSG298, pHSG396, and the like that have been altered to enhance copy numbers; and pACYC177, pACYC184, and the like that have a replicon derived from p15A; and, furthermore, plasmids derived from pSC101, ColE1, R1, F factor, and the like.

Furthermore, fusion protein-expressing vectors that enable easier purification such as pGEX-2T, pGEX-3X, pMal-c2 may be used. One example of the gene used as the starting material of the present invention has been described in Japanese Unexamined Patent Publication No. 7-308193.

Furthermore, in addition to plasmids, virus vectors

such as  $\lambda$  phage or M13 phage, or transposon may be used for the transformation of genes. With regard to the transformation of the gene into microorganisms other than Escherichia coli, gene transformation into organisms of 5 genus Bacillus by pUB110 (commercially available from Sigma) or pHY300PLK (commercially available from Takara Shuzo) is known. These vectors are described in "Molecular Cloning" (J. Sambrook, E.F. Fritsch, and T. Maniatis, Cold Spring Harbor Laboratory Press) and 10 "Cloning Vector" (P.H. Pouwels, B.B. Enger, Valk, and W.J. Brammar, Elsevier), and catalogues of the manufacturers.

Integration of the DNA fragment encoding the prenyl diphosphate synthase and, where needed, the DNA fragment 15 having the function of regulating expression of the gene of said enzyme into these vectors can be performed by a known method using an appropriate restriction enzyme and ligase. Specific examples of the plasmids thus constructed include, for example, pBs-SacGGPS.

As the microorganisms into which genes can be 20 directly introduced using such recombinant vectors include Escherichia coli and microorganisms of the genus Bacillus. Such transformation can also be carried out using general method, for example the  $\text{CaCl}_2$  method and 25 the protoplast method as described in "Molecular Cloning" (J. Sambrook, E.F. Fritsch, and T. Maniatis, Cold Spring Harbor Laboratory Press) and "DNA Cloning" Vol. I to III (D.M. Clover ed., IRL PRESS).

In order to produce the mutant enzyme of the present 30 invention, a host transformed as above is cultured, and then said culture is subjected to any method comprising salting out, precipitation with an organic solvent, gel chromatography, affinity chromatography, hydrophobic chromatography, ion exchange chromatography, and the like 35 to recover and purify said enzyme.

The present invention also provides a process for

producing farnesyl diphosphates using the enzyme of the present invention. According to this process, the enzyme of the present invention is reacted with a substrate in a medium, particularly an aqueous medium, and then, as desired, the prenyl diphosphate is harvested from the reaction medium. As the enzyme, not only a purified enzyme but also a crude enzyme that may be semi-purified to various stages, or a mixture of the cultured broth of a microorganism may be used. Alternatively there may be used immobilized enzymes prepared according to the general method from said enzyme, said crude enzyme, or a product containing the enzyme.

As the substrate, there may be used dimethyl allyl diphosphates or geranyl diphosphates and isopentenyl diphosphates. As the reaction medium, water or an aqueous buffer solution, for example Tris buffer or phosphate buffer and the like, may be used.

By using the method of producing the mutant prenyl diphosphate synthase obtained by the present invention, the mutant prenyl diphosphate synthase derived from a archaea may be created that is more stable and thus easier to handle and that produces prrenyl diphosphate. Furthermore, there is also expected a creation of the farnesyl diphosphate-producing mutant prenyl diphosphate synthase that has the property of the prenyl diphosphate synthase prior to mutation (for example, salt stability or stability in a wide range of pH) added thereto.

In the claims and the specification of the present invention, amino acid residues are expressed by the one-letter codes or three-letter codes as described hereinbelow:

A; Ala; alanine  
C; Cys; cysteine  
D; Asp; aspartic acid  
E; Glu; glutamic acid  
F; Phe; phenylalanine  
G; Gly; glycine

H; His; histidine  
I; Ile; isoleucine  
K; Lys; lysine  
L; Leu; leucine  
5 M; Met; methionine  
N; Asn; asparagine  
P; Pro; proline  
Q; Gln; glutamine  
R; Arg; arginine  
10 S; Ser; serine  
T; Thr; threonine  
V; Val; valine  
W; Trp; tryptophan  
Y; Tyr; tyrosine

15 Substitution of amino acid is expressed in the order  
of "the amino acid residue before substitution," "number  
of the amino acid residue," and "the amino acid residue  
after substitution," by the one-letter codes of amino  
acids. For example, the mutation in which a tyrosine  
20 residue at position 81 is replaced with a methionine  
residue is expressed as Y81M. Furthermore, the insertion  
of amino acid residues is expressed by "the number of the  
amino acid residue at the N-terminal side of the  
insertion site prior to insertion," "the amino acid  
25 residue that was inserted," and "the number of the amino  
acid residue at the C-terminal side of the insertion site  
prior to insertion." For example, the insertion of  
alanine in between the amino acid at position 84 and the  
amino acid at position 85 is expressed as 84A85.

30 EXAMPLES

The present invention is now explained with  
reference to specific examples, but they must not be  
construed to limit the invention in any way.

Example 1. Construction of a plasmid containing the gene  
for geranylgeranyl diphosphate synthase

The gene for the geranylgeranyl diphosphate synthase  
(hereinafter referred to as SacGGPS) derived from

Sulfolobus acidocaldarius was subcloned at the HindIII site of the plasmid vector pBluescript II (KS+) commercially available from Toyoboseki. The plasmid DNA was designated as pBs-SacGGPS. The SacGGPS gene is 5 available from Escherichia coli DH5 $\alpha$  (pGGPS1) that was internationally deposited on January 31, 1994 with the National Institute of Bioscience and Human Technology Agency of Industrial Science and Technology, of Ibalaki, Japan under the accession number of FERM BP-4982.

10 Also, the entire nucleotide sequence of the SacGGPS gene has been published in Japanese Unexamined Patent Publication No. 7-308193 Shin-ichi Ohnuma et al. (1994) The Journal of Biological Chemistry Vol. 269:14792-14797, or in the genetic information data bank such as GenBank 15 under the accession number D28748. Since Sulfolobus acidocaldarius is also available from various depositories of microorganisms such as ATCC etc. (as ATCC No. 33909), the DNA of the gene region of SacGGPS can be obtained by the conventional gene cloning method.

20 Example 2. Synthesis of the oligonucleotides for introducing mutation

For introducing mutation of the gene of geranylgeranyl diphosphate synthase, the following oligonucleotides were designed and synthesized:

25 Primer DNA (T78F, H81A):

5'-CATACTTTTCCTTGTGGCTGATGATATCATGGATC-3' (SEQ ID No: 3)

Primer DNA (T78F, H81L):

5'-CATACTTTTCCTTGTGCTTGATGATATCATGGATC-3' (SEQ ID No: 4)

Primer DNA (F77Y, T78F, H81L):

5'-CATACTTATTCCTTGTGGCTGATGATATCATGGATC-3' (SEQ ID No: 5)

Primer DNA (F77Y, T78F, H81A):

5'-CATACTTATTCCTTGTGGCTGATGATATCATGGATC-3' (SEQ ID No: 6)

Primer DNA (F77Y, T78S, V80I, I84L, 84PS85):

5'-GTTCTTCATACTTATTGCTTATTGATGATAGTATT-3' (SEQ ID No: 7), and 5'-ATTCATGATGATCTTCATCGATGGATCAAGAT-3' (SEQ ID No: 8).

Introduction of the mutation (F77Y, T78S, V80I, I84L, 84PS85) was effected using two nucleotides. First, mutation was introduced as mentioned in Example 3 using the oligonucleotide

5'-GTTCTTCATACTTATTGCTTATTGATGATAGTATT-3' (SEQ ID No: 7) and a transformant was prepared in accordance with Example 4, and furthermore mutation was introduced into the plasmid thus obtained using the oligonucleotide 5'-ATTCATGATGATCTTCATCGATGGATCAAGAT--3' (SEQ ID No: 8).

These nucleotides have a mutation in the codon encoding at least one amino acid residue selected from phenylalanine at position 77, threonine at position 78, valine at position 80, histidine at position 81, and isoleucine at position 84 in SacGGPS. In addition to the introduction of the codon encoding an amino acid that has been inserted in between isoleucine at position 84 and methionine at position 85, they are designed to newly introduce the cleavage site of the restriction enzyme BspHI (5'TGATGA3'), the cleavage site of the restriction enzyme EcoRV (5'GATATC3'), or the cleavage site of the restriction enzyme ClaI (5'ATCGAT3'). In the introduction of the cleavage site of BspHI, the amino acid sequence encoded by the SacGGPS gene does not change due to degeneracy of codons, or it is a site for an introduction of mutation. This is used to detect the substitution-mutated plasmid by means of agarose gel electrophoresis after digestion with an appropriate restriction enzyme, since the introduction of mutation by substitution into the SacGGPS gene simultaneously produces new cleavage sites of restriction enzymes.

These primer DNA's were subjected to phosphorylation treatment at 37°C for 30 minutes in the reaction medium shown below followed by denaturation at 70°C for 10 minutes:

10 pmol/μl primer DNA	2 μl
10 x kination buffer	1 μl
10 mM ATP	1 μl
H2O	5 μl
5 T4 polynucleotide kinase	1 μl

wherein the 10 x kination buffer is 1000 mM Tris-Cl (pH 8.0), 100 mM MgCl<sub>2</sub>, and 70 mM DTT.

Example 3. The introduction of substitution-mutation of the SacGGPSS gene

10 Using each primer DNA constructed in Example 2, substitution-mutation was introduced into the plasmid prepared in Example 1 in accordance with the Kunkel method. Mutan-K kit commercially available from Takara Shuzo was used to perform the Kunkel method. The 15 experimental procedure was as described in the kit insert. The substitution-mutation of the plasmid need not be conducted by the Kunkel method. For example, an identical result can be obtained by a method using the polymerase chain reaction (PCR).

20 Using Escherichia coli CJ236 in the Mutan-K kit as the host cell, a single strand DNA was obtained in which a thymine base in plasmid pBS-SacGGPS was replaced with a deoxyuracil base.

25 The single strand DNA thus obtained was used as the template in the reaction in which a primer DNA for synthesizing a complementary strand was treated in the following reaction solution at 65°C for 15 minutes and then annealed by allowing to stand at 37°C for 15 minutes:

30 Single strand DNA	0.6 pmol
Annealing buffer solution	1 μl
Primer DNA solution (Example 2)	1 μl
H2O make to a final volume of 10 μl	

in which the annealing buffer solution is 200 mM Tris-Cl (pH 8.0), 100 mM MgCl<sub>2</sub>, 500 mM NaCl, and 10 mM DTT.

Furthermore, 25 μl of the elongation buffer

5 solution, 60 units of Escherichia coli DNA ligase, and 1 unit of T4 DNA polymerase were added to synthesize the complementary strands at 25°C for 2 hours. The elongation buffer solution is 50 mM Tris-Cl (pH 8.0), 60 mM ammonium acetate, 5 mM MgCl<sub>2</sub>, 5 mM DTT, 1 mM NAD, and 0.5 mM dNTP.

10 After the reaction is over, 3  $\mu$  l of 0.2 M EDTA (pH 8.0) was added thereto and was subjected to treatment at 65°C for 5 minutes to stop the reaction.

15 Example 4. Construction of a recombinant having a gene in which substitution-mutation has been introduced into the SacGGPS gene

20 The DNA solution constructed in accordance with Example 3 was used to transform Escherichia coli XL1-Blue by the CaCl<sub>2</sub> method. An alternative method such as electroporation gives a similar result. A host cell other than Escherichia coli XL1-Blue, for example JM109 and the like also gave a similar result.

25 The transformant obtained by the CaCl<sub>2</sub> method was plated onto the agar plate containing ampicillin, a selectable marker of transformants, and was incubated overnight at 37°C.

30 Of the transformants obtained as above, the substitution-mutated pBs-SacGGPS plasmid that has a cleavage site of BspHI, EcoRV or ClaI was selected. The nucleotide sequence in the neighborhood of the codon corresponding to the amino acid residue that undergoes mutation of the SacGGPS gene of the selected substitution-mutated pBs-SacGGPS plasmid was determined by the dideoxy method. As a result, the pBs-SacGGPS plasmid containing the following five mutated SacGGPS genes was obtained. The nucleotide sequences encoding the amino acid sequences from the amino acid at position 77 to the amino acid at position 85 is shown below::

35 Mutation Nucleotide sequence  
T77F, H81A: 5'-TTTTCCCTGTGGCTGATGATATCATG-3' (SEQ ID No:

9)

T78F, H81L: 5'-TTTTCCCTTGCTTGATGATATCATG-3' (SEQ ID No: 10)

5 F77Y, T78F, H81L: 5'-TATTCCTTGCTTGATGATATCATG-3' (SEQ ID No: 11)

F77Y, T78F, H81A: 5'-TATTCCTTGCTGGCTGATGATATCATG-3' (SEQ ID No: 12)

F77Y, T78S, V80I, I84L, 84PS85:

5' -TATTCGCTTATTGATGATCTTCATCGATG-3' (SEQ ID No: 13)

10 Wild type: 5'-TTTACGCTTGTGCATGATGATATTATG-3' (SEQ ID No: 14).

Example 5. Measurement of activity of the mutant prenyl diphosphate synthase

Crude enzyme solutions were prepared as follows from 15 6 transformants comprising 5 mutant SacGGPS genes and one wild type SacGGPS gene obtained in Example 4.

The transformant cultured overnight in the 2 x LB medium was centrifuged to harvest cells, and then the 20 cells were suspended into a buffer for cell homogenization (50 mM calcium phosphate buffer solution (pH 5.8), 10 mM  $\beta$ -mercaptoethanol, 1 mM EDTA). This was homogenized by sonication and then centrifuged at 4°C at 10,000 r.p.m. for 10 minutes. The supernatant obtained was treated at 55°C for 12 hours to inactivate the 25 activity of prenyl diphosphate synthase derived from Escherichia coli. This was further centrifuged under the same condition and the supernatant obtained was used as a crude enzyme extract. When thermo stability was investigated the enzyme extract was incubated at 60°C, 30 70°C, or 80°C (60°C, 65°C, 67°C, or 70°C for the enzymes derived from Bacillus stearothermophilus) for one hour prior to reaction. The reaction was conducted at 55°C for 15 minutes in the following reaction solution:

[1-<sup>14</sup>C]-isopentenyl diphosphate (1 Ci/mol) 25 nmol

35 Allylic diphosphate (geranyl diphosphate) 25 nmol

Potassium phosphate buffer (pH 5.8) 10 mM

MgCl <sub>2</sub>	5 mM
Enzyme solution	100 µg
H <sub>2</sub> O	to make 200 µl

After the reaction is over, 200 µl of saturated NaCl  
5 was added to the reaction solution and 1 ml of  
water-saturated butanol was added thereto, which was then  
agituated, centrifuged, and separated into two phases. To  
800 µl of the butanol layer obtained was added 3 ml of a  
10 liquid scintillator and then the radioactivity was  
measured by the scintillation counter. The result is  
shown in Fig. 2.

The mutant prenyl diphosphate synthase has exhibited  
a thermo stability which is equal to that of the native  
15 geranylgeranyl diphosphate synthase, and is higher than  
that of the farnesyl diphosphate synthase derived from  
Bacillus stearothermophilus.

The solvent is evaporated from the remainder of the  
butanol layer by purging nitrogen gas thereinto while  
heating the layer in order to concentrate to a volume of  
20 about 0.5 ml. To the concentrate were added 2 ml of  
methanol and one ml of potato acid phosphatase solution  
(2 mg/ml potato acid phosphatase, 0.5 M sodium acetate  
(pH 4.7)) to effect the dephosphorylation reaction at  
37°C. Subsequently the dephosphorylated reaction product  
25 was extracted with 3 ml of n-pentane.

This was concentrated by evaporating the solvent by  
purging nitrogen gas thereinto, which was then analyzed  
by TLC (reverse phase TLC plate: LKC18 (Whatman),  
development solvent: acetone/water = 9/1). The developed  
30 dephosphorylated reaction product was analyzed by the Bio  
Image Analyzer BAS2000 (Fuji Photo Film) to determine the  
location of radioactivity. The result when geranyl  
diphosphate was used as the allylic substrate is shown in  
Fig. 3.

35 The reaction product of the mutant prenyl  
diphosphate synthase was shown to be a farnesyl

diphosphate.

SEQUENCE LISTING

SEQ ID No: 1

LENGTH: 330

TYPE: amino acid

TOPOLOGY: linear

MOLECULE TYPE: protein

ORIGINAL SOURCE:

ORGANISM: Sulfolobus acidocaldarius

STRAIN: ATCC 33909

FEATURE:

FEATURE KEY: Asp-rich domain

LOCATION: 82-86

Met Ser Tyr Phe Asp Asn Tyr Phe Asn Glu Ile Val Asn Ser Val Asn  
5 10 15  
Asp Ile Ile Lys Ser Tyr Ile Ser Gly Asp Val Pro Lys Leu Tyr Glu  
20 25 30  
Ala Ser Tyr His Leu Phe Thr Ser Gly Gly Lys Arg Leu Arg Pro Leu  
35 40 45  
Ile Leu Thr Ile Ser Ser Asp Leu Phe Gly Gly Gln Arg Glu Arg Ala  
50 55 60  
Tyr Tyr Ala Gly Ala Ala Ile Glu Val Leu His Thr Phe Thr Leu Val  
65 70 75 80  
His Asp Asp Ile Met Asp Gln Asp Asn Ile Arg Arg Gly Leu Pro Thr  
85 90 95  
Val His Val Lys Tyr Gly Leu Pro Leu Ala Ile Leu Ala Gly Asp Leu  
100 105 110  
Leu His Ala Lys Ala Phe Gln Leu Leu Thr Gln Ala Leu Arg Gly Leu  
115 120 125  
Pro Ser Glu Thr Ile Ile Lys Ala Phe Asp Ile Phe Thr Arg Ser Ile  
130 135 140  
Ile Ile Ile Ser Glu Gly Gln Ala Val Asp Met Glu Phe Glu Asp Arg  
145 150 155 160  
Ile Asp Ile Lys Glu Gln Glu Tyr Leu Asp Met Ile Ser Arg Lys Thr  
165 170 175  
Ala Ala Leu Phe Ser Ala Ser Ser Ile Gly Ala Leu Ile Ala Gly  
180 185 190

Ala Asn Asp Asn Asp Val Arg Leu Met Ser Asp Phe Gly Thr Asn Leu  
195 200 205  
Gly Ile Ala Phe Gln Ile Val Asp Asp Ile Leu Gly Leu Thr Ala Asp  
210 215 220  
Glu Lys Glu Leu Gly Lys Pro Val Phe Ser Asp Ile Arg Glu Gly Lys  
225 230 235 240  
Lys Thr Ile Leu Val Ile Lys Thr Leu Glu Leu Cys Lys Glu Asp Glu  
245 250 255  
Lys Lys Ile Val Leu Lys Ala Leu Gly Asn Lys Ser Ala Ser Lys Glu  
260 265 270  
Glu Leu Met Ser Ser Ala Asp Ile Ile Lys Lys Tyr Ser Leu Asp Tyr  
275 280 285  
Ala Tyr Asn Leu Ala Glu Lys Tyr Tyr Lys Asn Ala Ile Asp Ser Leu  
290 295 300  
Asn Gln Val Ser Ser Lys Ser Asp Ile Pro Gly Lys Ala Leu Lys Tyr  
305 310 315 320  
Leu Ala Glu Phe Thr Ile Arg Arg Arg Lys  
325 330

SEQ ID No: 2

LENGTH: 993

TYPE: nucleic acid

STRANDEDNESS: double

TOPOLOGY: linear

MOLECULE TYPE: genomic DNA

ORIGINAL SOURCE:

ORGANISM: Sulfolobus acidocaldarius

STRAIN: ATCC 33909

FEATURE: Feature key: Asp-rich domain coding

LOCATION: 246-258

ATGAGTTACT TTGACAACTA TTTTAATGAG ATTGTTAATT CTGTAAACGA CATTATTAAG 60  
AGCTATATAT CTGGAGATGT TCCTAAACTA TATGAAGCCT CATATCATTT GTTTACATCT 120  
GGAGGTAAGA GGTAAAGACC ATTAATCTTA ACTATATCAT CAGATTATT CGGAGGACAG 180  
AGAGAAAGAG CTTATTATGC AGGTGCAGCT ATTGAAGTTC TTCATACTTT TACGCTTG 240  
CATGATGATA TTATGGATCA AGATAATATC AGAAGAGGGT TACCCACAGT CCACGTGAAA 300  
TACGGCTTAC CCTTAGCAAT ATTAGCTGGG GATTTACTAC ATGCAAAGGC TTTTCAGCTC 360  
TTAACCCAGG CTCTTAGAGG TTTGCCAAGT GAAACCATAA TTAAGGCTTT CGATATTTTC 420  
ACTCGTTCAA TAATAATTAT ATCCGAAGGA CAGGCAGTAG ATATGGAATT TGAGGACAGA 480

ATTGATATAA AGGAGCAGGA ATACCTTGAC ATGATCTCAC GTAAGACAGC TGCATTATTC 540  
TCGGCATCCT CAAGTATAAGG CGCACTTATT GCTGGTGCTA ATGATAATGA TGTAAGACTG 600  
ATGTCTGATT TCGGTACGAA TCTAGGTATT GCATTTCAGA TTGTTGACGA TATCTTAGGT 660  
CTAACAGCAG ACGAAAAGGA ACTTGGAAAG CCTGTTTTA GTGATATTAG GGAGGGTAAA 720  
AAGACTATAC TTGTAATAAA AACACTGGAG CTTTGTAAAG AGGACGAGAA GAAGATTGTC 780  
CTAAAGGCGT TAGGTAATAA GTCAGCCTCA AAAGAAGAAT TAATGAGCTC AGCAGATATA 840  
ATTAAGAAAT ACTCTTTAGA TTATGCATAC AATTAGCAG AGAAATATTA TAAAAATGCT 900  
ATAGACTCTT TAAATCAAGT CTCCCTCAAG AGTGATATAC CTGGAAAGGC TTTAAAATAT 960  
CTAGCTGAAT TTACGATAAG AAGGAGAAAA TAA

SEQ ID No: 3

LENGTH: 37

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: synthetic DNA

SEQUENCE DESCRIPTION:

CATACTTTT TCCTTGTGGC TGATGATATC ATGGATC

37

SEQ ID No: 4

LENGTH: 37

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: synthetic DNA

SEQUENCE DESCRIPTION:

CATACTTTT TCCTTGTGCT TGATGATATC ATGGATC

37

SEQ ID No: 5

LENGTH: 37

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: synthetic DNA

SEQUENCE DESCRIPTION:

CATACTTATT TCCTTGTGCT TGATGATATC ATGGATC

37

SEQ ID No: 6

LENGTH: 37

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear  
MOLECULE TYPE: synthetic DNA  
SEQUENCE DESCRIPTION:  
CATACTTATT TCCTTGTGGC TGATGATATC ATGGATC 37  
SEQ ID No: 7  
LENGTH: 36  
TYPE: nucleic acid  
STRANDEDNESS: single  
TOPOLOGY: linear  
MOLECULE TYPE: synthetic DNA  
SEQUENCE DESCRIPTION:  
GTTCTTCATA CTTATTCGCT TATTCATGAT AGTATT 36  
SEQ ID No: 8  
LENGTH: 33  
TYPE: nucleic acid  
STRANDEDNESS: single  
TOPOLOGY: linear  
MOLECULE TYPE: synthetic DNA  
SEQUENCE DESCRIPTION:  
ATTCATGATG ATCTTCCATC GATGGATCAA GAT 33  
SEQ ID No: 9  
LENGTH: 27  
TYPE: nucleic acid  
STRANDEDNESS: single  
TOPOLOGY: linear  
MOLECULE TYPE:  
SEQUENCE DESCRIPTION:  
TTTTTCCTTG TGGCTGATGA TATCATG 27  
SEQ ID No: 10  
LENGTH: 27  
TYPE: nucleic acid  
STRANDEDNESS: single  
TOPOLOGY: linear  
MOLECULE TYPE:  
SEQUENCE DESCRIPTION:  
TTTTTCCTTG TGCTTGATGA TATCATG 27  
SEQ ID No: 11

LENGTH: 27

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE:

SEQUENCE DESCRIPTION:

TATTCCTTG TGCTTGATGA TATCATG

27

SEQ ID No: 12

LENGTH: 27

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE:

SEQUENCE DESCRIPTION:

TATTCCTTG TGGCTGATGA TATCATG

27

SEQ ID No: 13

LENGTH: 33

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE:

SEQUENCE DESCRIPTION:

TATTCGCTTA TTCATGATGA TCTTCCATCG ATG

33

SEQ ID No: 14

LENGTH: 27

TYPE: nucleic acid

STRANDEDNESS: single

TOPOLOGY: linear

MOLECULE TYPE:

SEQUENCE DESCRIPTION:

TTTACGCTTG TGCATGATGA TATTATG

27

CLAIMS

1. A mutant prenyl diphosphate synthase having a modified amino acid sequence, wherein

at least one amino acid residue selected

5 from (a) the amino acid residues in between the amino acid residue located at the fifth position in the N-terminal direction from D of the N-terminal and the amino acid residue located at the first position in the N-terminal direction from D of said N-terminal of the

10 aspartic acid-rich domain DDXX(XX)D (wherein X denotes any amino acid, and the two X's in the parentheses may not be present) present in region II, and (b) the amino acid residue located at the first position in the N-terminal direction from D of the C-terminal of said aspartic acid-rich domain has been substituted by another

15 amino acid, and/or

20 additional amino acid(s) have been inserted in between the amino acid residue located at the first position in the N-terminal direction from D of the C-terminal and D of the C-terminal of said aspartic acid-rich domain.

25 2. A mutant enzyme according to claim 1 wherein the reaction product of the prenyl diphosphate synthase is farnesyl diphosphate.

30 3. A mutant enzyme according to claim 1 wherein the prenyl diphosphate synthase is of the homodimer-type.

4. A mutant enzyme according to claim 1 wherein the prenyl diphosphate synthase is derived from archaea.

35 5. A mutant enzyme according to claim 1 wherein the prenyl diphosphate synthase is derived from Sulfolobus acidocaldarius.

6. A mutant enzyme according to claim 1 wherein the prenyl diphosphate synthase retains the properties that were owned by the prenyl diphosphate synthase prior to mutation.

35 7. A mutant enzyme according to claim 1 wherein the prenyl diphosphate synthase is a thermostable enzyme.

8. A mutant prenyl diphosphate synthase according to claim 1, wherein at least one amino acid selected from phenylalanine at position 77, threonine at position 78, valine at position 80, histidine at position 81, and isoleucine at position 84 has been substituted by another amino acid, or one or more amino acids have been inserted in between isoleucine at position 84 and methionine at position 85 in the geranylgeranyl diphosphate synthase as set forth in SEQ ID No: 1.

10 9. A mutant prenyl diphosphate synthase according to claim 1 wherein at least one amino acid selected from phenylalanine at position 77, threonine at position 78, valine at position 80, histidine at position 81, and isoleucine at position 84 has been substituted by another amino acid, and/or two amino acids have been inserted in 15 between isoleucine at position 84 and methionine at position 85 in the geranylgeranyl diphosphate synthase as set forth in SEQ ID NO: 1, wherein the phenyl alanine at position 77 has been replaced with tyrosine, the threonine at position 78 has been replaced with 20 phenylalanine or serine, the valine at position 80 has been replaced with isoleucine, the histidine at position 81 has been replaced with leucine or alanine, or the isoleucine at position 84 has been replaced with leucine; or proline and serine have been inserted in 25 between the isoleucine at position 84 and the methionine at position 85.

10. A mutant prenyl diphosphate synthase according to claim 1, wherein the mutant prenyl diphosphate synthase is derived from a native geranylgeranyl diphosphate synthase of an organism selected from the group consisting of Arabidopsis thaliana, Lupinus albus, Capsicum annuum, Sulfolobus acidocaldarius, Rhodobacter sphaeroides, Rhodobacter capsulatus, Erwinia herbicola, Myxococcus thaliana and Neurospora crassa.

30 11. A DNA encoding an enzyme according to claim 1.

35 12. An RNA transcribed from a DNA according to

claim 11.

13. A recombinant vector comprising a DNA according to claim 11.

5 14. A host organism transformed with a recombinant vector according to claim 13.

10 15. A process for producing a mutant enzyme according to claim 1, said method comprising the steps of culturing a host transformed with an expression vector comprising a DNA coding for the mutant enzyme and of harvesting the expression product from the culture.

15 16. A process for producing a prenyl diphosphate having not more than 15 carbons comprising the step of bringing an enzyme according to any of claims 1 to 10 or an enzyme produced by the method according to claim 15 into contact with a substrate selected from the group consisting of isopentenyl diphosphate, dimethylallyl diphosphate, and geranyl diphosphate.

PCT/GB2002/002204

FARNESYL DIPHOSPHATE SYNTHASE

5

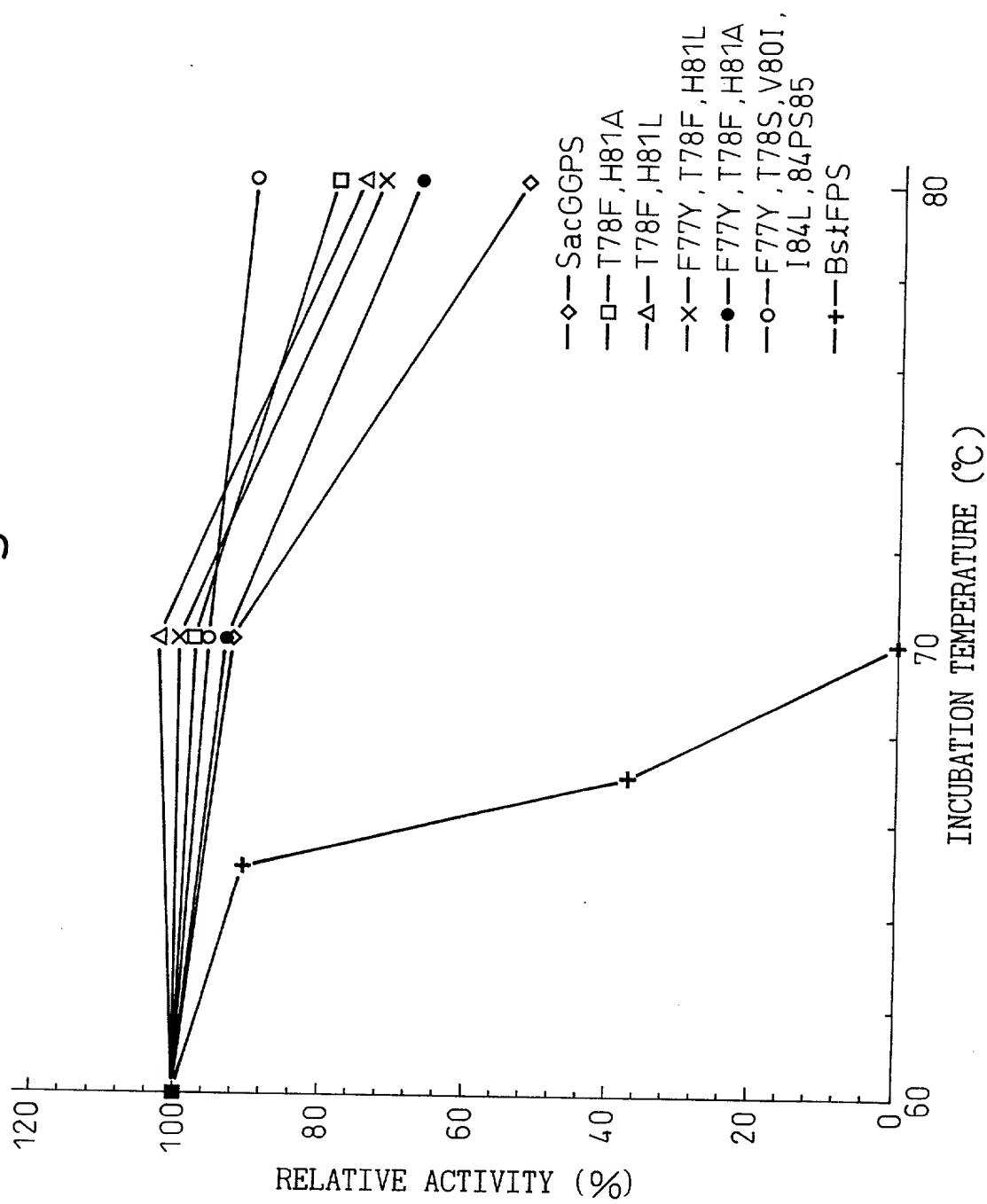
ABSTRACT OF DISCLOSURE

10 A mutant prenyl diphosphate synthase capable of synthesizing prenyl diphosphates, shorter than those synthesized by the original enzyme, by modifying the amino acid sequence in and upstream of the aspartic acid-rich domain DDXX(XX)D (X denotes any amino acid, and XX in the parentheses may not be present) present in region II of the prenyl diphosphate synthase.

# Fig. 1

REGION I		REGION II		REGION III	
ATGERPYRS	116 GGKVR	147 EMIHTMSLIH	DILPCMDNDLRRG	238 GQVVD	
LA15778.p	110 . . . . .	141 . . . . .		230 . . . . .	
CAGERDIS.	118 . . . . .	149 . . . . .		238 . . . . .	
ATGGPSRP.	88 . P . AP	119 . V . AA		211 . Y . .	
GGPS.pep	43 . . . L .	74 . VL . FT . V	— I . Q . NI . .	160 . A . .	
SPCRT.pep	64 . A . I .	95 . L . CA . V	— F . DAEI . .	185 . GWE	
RCPHSYNG.	192 . A . I .	223 . LM . CA . V	— AF . A . I .	313 . AWE	
EHCRTS.pe	54 . . . I .	86 . LT . A . ML	— AE . . .	175 . FR .	
MXCRTNODA	104 . . . L .	136 . LL . FL .	— VA . QAE . .	199 . YL .	
NCAL3.pep	197 . . . DI .	226 . L . A . LV	— VE . SV . .	260 . GM .	
REGION IV		REGION V		REGION VI	
ATGERPYRS	265 KT	293 GLLFQVVDIL_DVTKSSK_ELGKTAGKDLIADK			
LA15778.p	255 . .	283 . M . . . V . —			
CAGERDIS.	263 . .	291 . . . . .	— E . . . . .		
ATGGPSRP.	230 . F	256 . M . Y . . . .	TE . KK . YDGGAE		
GGPS.pep	185 . .	211 . IA . . I . .	GMMEAEL .		
SPCRT.pep	203 . .	227 . EA . . A . .	GLTADEKE . .		
RCPHSYNG.	331 . .	355 . SA . . IA . .	PVFS . IREG .		
EHCRTS.pe	199 . .	225 . QA . . LL . .	LR . HPET . .		
MXCRTNODA	226 . .	247 . AY . LR . . L	GLFGD . NV . A .		
NCAL3.pep	286 . .	272 . I . . IA . .	YHNLWNREYT . AN . GMCE . . TEG .		

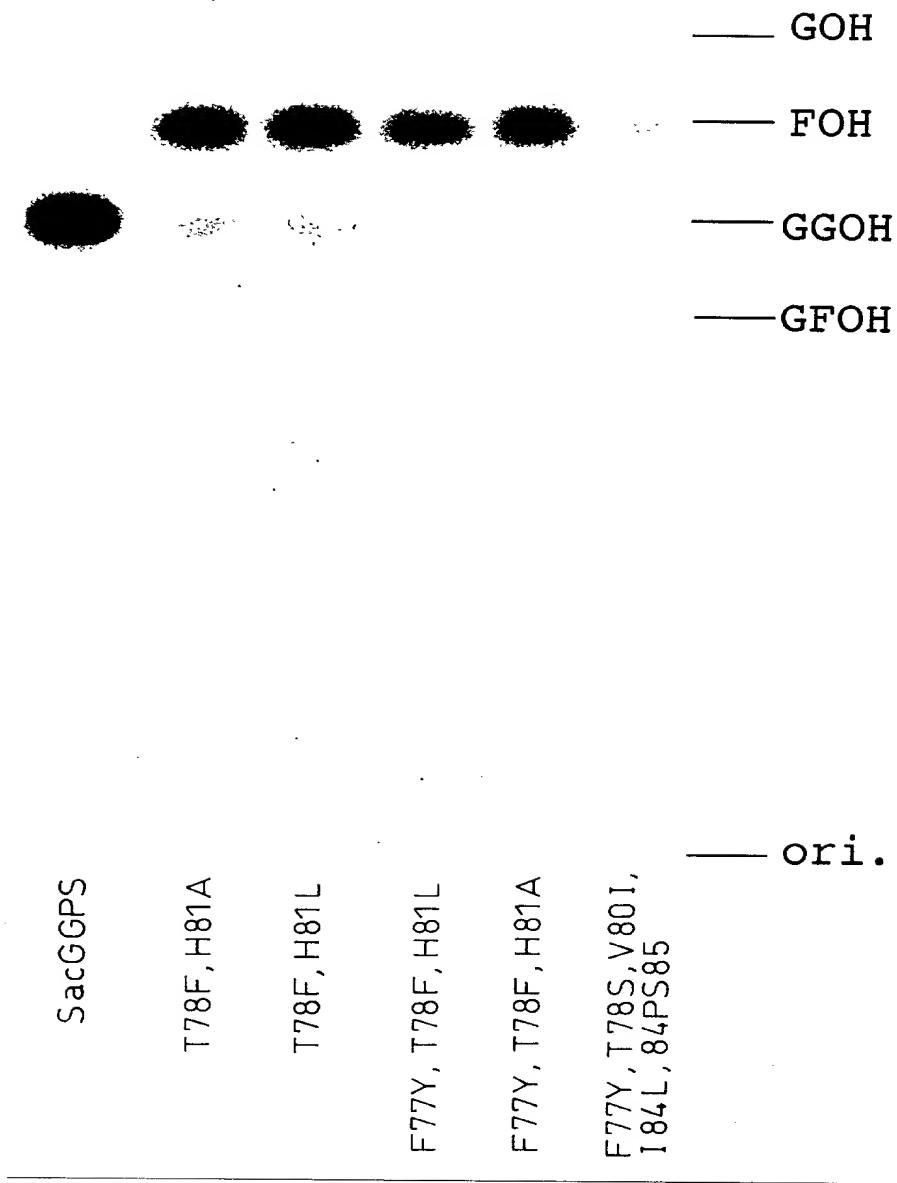
Fig. 2



3/3

Fig. 3

— s.f.



BOX: SEQUENCE  
Docket No. 77670/495

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Inventors: Hiroyuki NAKANE, et al.

Serial No.: -- Not Yet Assigned --

Art Unit: -- Not Yet Assigned --

Filing Date: -- Concurrent Herewith --

For: FARNESYL DIPHOSPHATE SYNTHASE

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Box Sequence  
Washington, DC 20231

**VERIFICATION STATEMENT ACCOMPANYING TRANSMITTAL  
OF SEQUENCE LISTING**

Sir:

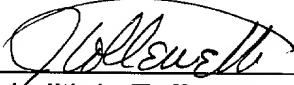
In accord with 37 CFR §1.821(c) and (e), Applicants hereby submit a substitute paper copy of a Sequence Listing to replace that originally contained in the specification filed concurrently herewith. A computer readable copy of the Sequence Listing is also included.

In accord with 37 CFR §1.821(f) the undersigned Attorney verifies that the paper copy and the computer readable copy of the substitute Sequence Listing are identical, and no new matter is included.

Respectfully submitted,

Date July 22, 1997

By

  
Judith L. Toffenetti  
Reg. No. 39,048

KENYON & KENYON  
1025 Connecticut Avenue, NW  
Washington, DC 20036  
telephone: 202/429-1776  
facsimile: 202/429-0796

137498

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANTS: Hiroyuki NAKANE, Chikara OHTO, Shinichi OHNUMA, Kazutake HIROOKA, Tokuzo NISHINO

(ii) TITLE OF INVENTION: Farnesyl Diphosphate Synthase

(iii) NUMBER OF SEQUENCES: 14

(iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: Kenyon & Kenyon
- (B) STREET: One Broadway
- (C) CITY: New York
- (D) STATE: NY
- (E) COUNTRY: USA
- (F) ZIP: 10004

(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: 3½ Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS 6.2
- (D) SOFTWARE: WordPerfect 6.1 Windows

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER: Not Yet Issued
- (B) FILING DATE: Concurrent Herewith
- (C) CLASSIFICATION: Not Yet Issued

(vii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: JP 8-213211
- (B) FILING DATE: 24-JUL-96

(viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Edward W. Greason
- (B) REGISTRATION NUMBER: 18,918
- (C) REFERENCE/DOCKET NUMBER: 77670/495

(ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: (212)425-7200
- (B) TELEFAX: (212)425-5288

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 330 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Sulfolobus acidocaldarius*
- (B) STRAIN: ATCC 33909

(ix) FEATURE:

- (A) KEY: Asp-rich domain
- (B) LOCATION: 82-86

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Ser Tyr Phe Asp Asn Tyr Phe Asn Glu Ile Val Asn Ser Val Asn  
5 10 15  
Asp Ile Ile Lys Ser Tyr Ile Ser Gly Asp Val Pro Lys Leu Tyr Glu  
20 25 30  
Ala Ser Tyr His Leu Phe Thr Ser Gly Gly Lys Arg Leu Arg Pro Leu  
35 40 45  
Ile Leu Thr Ile Ser Ser Asp Leu Phe Gly Gly Gln Arg Glu Arg Ala  
50 55 60  
Tyr Tyr Ala Gly Ala Ala Ile Glu Val Leu His Thr Phe Thr Leu Val  
65 70 75 80  
His Asp Asp Ile Met Asp Gln Asp Asn Ile Arg Arg Gly Leu Pro Thr  
85 90 95  
Val His Val Lys Tyr Gly Leu Pro Leu Ala Ile Leu Ala Gly Asp Leu  
100 105 110  
Leu His Ala Lys Ala Phe Gln Leu Leu Thr Gln Ala Leu Arg Gly Leu  
115 120 125  
Pro Ser Glu Thr Ile Ile Lys Ala Phe Asp Ile Phe Thr Arg Ser Ile  
130 135 140  
Ile Ile Ile Ser Glu Gly Gln Ala Val Asp Met Glu Phe Glu Asp Arg  
145 150 155 160  
Ile Asp Ile Lys Glu Gln Glu Tyr Leu Asp Met Ile Ser Arg Lys Thr  
165 170 175  
Ala Ala Leu Phe Ser Ala Ser Ser Ile Gly Ala Leu Ile Ala Gly  
180 185 190  
Ala Asn Asp Asn Asp Val Arg Leu Met Ser Asp Phe Gly Thr Asn Leu  
195 200 205  
Gly Ile Ala Phe Gln Ile Val Asp Asp Ile Leu Gly Leu Thr Ala Asp  
210 215 220  
Glu Lys Glu Leu Gly Lys Pro Val Phe Ser Asp Ile Arg Glu Gly Lys  
225 230 235 240  
Lys Thr Ile Leu Val Ile Lys Thr Leu Glu Leu Cys Lys Glu Asp Glu

245	250	255
Lys Lys Ile Val Leu Lys Ala Leu Gly Asn Lys Ser Ala Ser Lys Glu		
260	265	270
Glu Leu Met Ser Ser Ala Asp Ile Ile Lys Lys Tyr Ser Leu Asp Tyr		
275	280	285
Ala Tyr Asn Leu Ala Glu Lys Tyr Tyr Lys Asn Ala Ile Asp Ser Leu		
290	295	300
Asn Gln Val Ser Ser Lys Ser Asp Ile Pro Gly Lys Ala Leu Lys Tyr		
305	310	315
Leu Ala Glu Phe Thr Ile Arg Arg Arg Lys		
325	330	

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 993 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: genomic DNA

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Sulfolobus acidocaldarius*
- (B) STRAIN: ATCC 33909

(ix) FEATURE:

- (A) KEY: Asp-rich domain coding
- (B) LOCATION: 246-258

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATGAGTTACT TTGACAACTA TTTAATGAG ATTGTTAATT CTGTAAACGA CATTATTAAG	60
AGCTATATAT CTGGAGATGT TCCTAAACTA TATGAAGCCT CATATCATTG GTTTACATCT	120
GGAGGTAAGA GGTTAAGACC ATTAATCTTA ACTATATCAT CAGATTATT CGGAGGACAG	180
AGAGAAAGAG CTTATTATGC AGGTGCAGCT ATTGAAGTTC TTCATACTTT TACGCTTGTG	240
CATGATGATA TTATGGATCA AGATAATATC AGAAGAGGGT TACCCACAGT CCACGTGAAA	300
TACGGCTTAC CCTTAGCAAT ATTAGCTGGG GATTTACTAC ATGCAAAGGC TTTTCAGCTC	360
TTAACCCAGG CTCTTAGAGG TTTGCCAAGT GAAACCATAA TTAAGGCTTT CGATATTTC	420
ACTCGTTCAA TAATAATTAT ATCCGAAGGA CAGGCAGTAG ATATGGAATT TGAGGACAGA	480

ATTGATATAA	AGGAGCAGGA	ATACCTTGAC	ATGATCTCAC	GTAAGACAGC	TGCATTATTC	540
TCGGCATTCT	CAAGTATAGG	CGCACTTATT	GCTGGTGCTA	ATGATAATGA	TGTAAGACTG	600
ATGTCTGATT	TCGGTACGAA	TCTAGGTATT	GCATTCAGA	TTGTTGACGA	TATCTTAGGT	660
CTAACACAGCAG	ACGAAAAGGA	ACTTGAAAG	CCTGTTTTA	GTGATATTAG	GGAGGGTAAA	720
AAGACTATAC	TTGTAATAAA	AACACTGGAG	CTTGTAAG	AGGACGAGAA	GAAGATTGTC	780
CTAAAGGCCT	TAGGTAATAA	GTCAGCCTCA	AAAGAAGAAT	TAATGAGCTC	AGCAGATATA	840
ATTAAGAAAT	ACTCTTTAGA	TTATGCATAC	AATTTAGCAG	AGAAATATTA	TAAAAATGCT	900
ATAGACTCTT	TAAATCAAGT	CTCCTCTAAG	AGTGATATAC	CTGGAAAGGC	TTTAAAATAT	960
CTAGCTGAAT	TTACGATAAG	AAGGAGAAAA	TAA			993

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH:37
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CATACTTTT TCCTTGTGGC TGATGATATC ATGGATC

37

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH:37
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CATACTTTT TCCTTGTGCT TGATGATATC ATGGATC

37

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 37
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CATACTTATT TCCTTGTGCT TGATGATATC ATGGATC

37

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 37
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CATACTTATT TCCTTGTGGC TGATGATATC ATGGATC

37

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GTTCTTCATA CTTATTCGCT TATTCATGAT AGTATT

36

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATTCATGATG ATCTTCCATC GATGGATCAA GAT

33

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TTTTCCTTG TGGCTGATGA TATCATG

27

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TTTTCCTTG TGCTTGATGA TATCATG

27

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TATTCCTTG TGCTTGATGA TATCATG

27

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

TATTCCTTG TGGCTGATGA TATCATG

27

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TATTCGCTTA TTCAATGATGA TCTTCCATCG ATG

33

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TTTACGCTTG TGCATGATGA TATTATG

27

# Declaration and Power of Attorney For Patent Application

## English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

FARNESYL DIPHOSPHATE SYNTHASE

the specification of which

(check one)

is attached hereto.

was filed on \_\_\_\_\_ as  
Application Serial No. \_\_\_\_\_  
and was amended on \_\_\_\_\_  
(if applicable)

hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)			Priority Claimed	
08-213211			<input checked="" type="checkbox"/> Yes	<input type="checkbox"/> No
(Pat. Appln.)	Japan	24/July/1996	<input checked="" type="checkbox"/>	<input type="checkbox"/>
(Number)	(Country)	(Day/Month/Year Filed)	Yes	No
(Number)	(Country)	(Day/Month/Year Filed)	<input type="checkbox"/>	<input checked="" type="checkbox"/>
(Number)	(Country)	(Day/Month/Year Filed)	<input type="checkbox"/>	<input checked="" type="checkbox"/>

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)

(Filing Date)

(Status)  
(patented, pending, abandoned)

(Application Serial No.)

(Filing Date)

(Status)  
(patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (list name and registration number)

Edward W. Greason Reg. No. 18,918

Send Correspondence to:

Edward W. Greason, Kenyon &amp; Kenyon, One Broadway, New York, N.Y. 10004

Direct Telephone Calls to: (name and telephone number)

Edward W. Greason (212) 425-7200

Full name of sole or first inventor

Hiroyuki Nakane

Inventor's signature

Hiroyuki Nakane

Date

June 20, 1997

Residence

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Full name of second joint inventor, if any

Chikara Ohto

Second Inventor's signature

Chikara Ohto

Date

June 20, 1997

Residence

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Citizenship

Japanese

Post Office Address

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(Supply similar information and signature for third and subsequent joint inventors.)

Full name of third joint inventor, if any		Shinichi Ohnuma
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Citizenship		Japanese
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Inventor's signature		<i>Tokuzo Nishino</i>
Residence		Sendai-shi, Miyagi, Japan
Citizenship		Japanese
Post Office Address		2-15-3, Minamiyoshinari, Aoba-ku, Sendai-shi, Miyagi, Japan
Full name of sixth joint inventor, if any		
Inventor's signature		Date
Residence		
Citizenship		
Post Office Address		
Full name of seventh joint inventor, if any		
Inventor's signature		Date
Residence		
Citizenship		
Post Office Address		